Two Criniviruses Are Associated with the Strawberry Pallidosis Disease

I.E. Tzanetakis and A.B. Halgren
Dept. of Botany and Plant Pathology
Oregon State University
Corvallis, 97331
USA

W.M. Wintermantel
USDA-ARS,
Salinas, CA 93905
USA

K.E. Keller and R.R. Martin
USDA-ARS
Corvallis, OR 97330
USA

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Abstract

Pallidosis, a disease attributed to a graft-transmissible agent is the focal point of this study. Two viruses belonging to the *Closteroviridae* family, genus *Crinivirus* that can cause pallidosis symptoms on strawberry indicator plants have been identified. A previously unidentified virus designated as *Strawberry pallidosis associated virus* (SPaV) is the predominant virus in pallidosis positive plants, while the second virus is *Beet pseudo-yellows virus* (BPYV). The genomes of both viruses have been sequenced fully and phylogenetic analyses indicate that the two viruses are more closely related than any other crinivirus found in the database. Epidemiological studies have demonstrated that the greenhouse whitefly, *Trialeuroides vaporariorum,* is an efficient vector of SPaV. Protocols for molecular detection of both viruses using reverse transcription-polymerase chain reaction (RT-PCR) have been developed. The recombinant major coat protein of SPaV has been expressed in bacteria and polyclonal antibodies to the virus have been developed that facilitate detection of the virus in tissue blot immunoassays (TBIA). The potential of seed and pollen transmission of SPaV was examined as an alternative mode of transmission of the virus. The geographical distribution of both viruses in the major strawberry producing regions of the United States has been examined.

INTRODUCTION

Pallidosis is a disease of strawberry first identified in 1957 in both Australia and the United States (Frazier and Stubbs, 1969). The disease has also been reported in East Canada, Arkansas and the Mid-Atlantic States (Craig, 1981; Fulton and Moore, 1982; Hokanson et al. 2000) while there is no information of its' distribution in other strawberry producing areas. Pallidosis is defined as a disease caused by a graft-transmissible agent that when grafted onto indicator plants *Fragaria virginiana* 'UC-10' or 'UC-11' causes marginal chlorosis and epinasty of the leaves (Fig. 1) while *F. vesca* 'UC-4' or 'UC-5' indicators remain asymptomatic. In studies performed in the state of Maryland (Hokanson et al. 2000) the pallidosis disease was widespread with more than 70% of the plants tested infected with the agent. These results indicated that the disease may have been an under-recognized problem in the strawberry industry due to the difficulty by which it is detected. Investigators (Henriques and Schlegel, 1975) were able to identify inclusion bodies similar to those of *Beet yellows virus* and (Yoshikawa and Converse, 1990) extracted dsRNA from pallidosis positive plants. This study identifies two viruses associated with the disease. A previously unidentified virus designated as *Strawberry pallidosis associated virus* (SPaV) and *Beet pseudo-yellows virus* (BPYV). Both viruses belong to the genus *Crinivirus*, family *Closteroviridae.* Criniviruses have bipartite genome and are known to be transmitted by one or more of four species of whiteflies belonging the genus *Trialeuroides* and *Bemisia* (Wisler et al., 1998).

MATERIALS AND METHODS

Virus sources for this work were 29 field isolates from Maryland, USA, 2 from California and 7 from the USDA-NCGR in Corvallis, Oregon. For all reactions described hereafter enzymes were from Invitrogen Corp. (Carlsbad, CA) and used according to the

manufacturer's recommendations unless otherwise noted. dsRNA was extracted and used for reverse transcription and cloning as described elsewhere (Tzanetakis et al., 2004) from a pallidosis positive field isolate (M1). Primers were developed from cloned sequences that had high homology to crinivirus sequences in the GenBank. dsRNA also was extracted, cloned and sequenced from isolate M29 that did not test positive with the SPaV primers in reverse transcription-polymerase chain reaction (RT-PCR).

RNA for RT-PCR was extracted as described previously (Hughes and Galau, 1988). Detection was performed with primers SP 44F/R that amplify a 517 bp fragment of the HSP70h of SPaV and BP CPm F/R that amplify a 334 bp fragment of the minor coat protein gene (CPm) of BPYV (Table 1).

For determination of the complete genome sequence of both viruses we performed one RT reaction and two PCR reactions for all parts of the genomes for which there were not at least three identical clones from the initial cloning reactions. The 3' ends of the virus genomes were obtained after polyadenylation of dsRNA (Tzanetakis and Martin, 2004) and performance of 3'RACE. The reactions for determining the 3'termini were performed twice. The 5' ends were determined using two approaches. Reverse primers were developed from the minus-strand of viral dsRNAs and were utilized in combination with oligo-thymidine primers utilizing adenylated dsRNA in RT-PCR. We also performed 5'RACE by tailing cDNA using a terminal transferase and deoxycytosine. Each of the alternative 5' RACE reactions were performed once.

Assembly of the consensus was performed utilizing the CLUSTAL W software at http://www.ebi.ac.uk/clustalw, while the search for transmembrane domains encoded proteins were performed using the TMHMM and SignalP Servers v. 2.0 (http://www. cbs.dtu.dk/services/). Identification of conserved domains utilizing the Conserved Domain Architecture Retrieval Tool at http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington. cgi?cmd=rps. Phylogenetic analysis was performed at http://www.genebee.msu.su (A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University).

Virus transmission studies were carried out using the greenhouse whitefly (*T. vaporariorum*). Transmissions were conducted in the laboratory in sealed cages. Insects were allowed extended feeding periods of 24 hours (BPYV) and 48 hours (SPaV) on strawberry plants positive for SPaV or BPYV, as confirmed by RT-PCR. Following acquisition of virus, whiteflies were transferred to virus free strawberry plants, or other plant species. After 48 hours, insects were removed from plants by spraying with the contact insecticide imidocloprid, and plants were transferred to the greenhouse. Mock inoculations were performed simultaneously to confirm whitefly stock populations remained virus free. At 6 weeks post-inoculation nucleic acid was extracted from leaf tissue and tested by RT-PCR for the presence of BPYV and SPaV to confirm infection.

Seed from 12 infected plants was germinated and tested after three to twelve months for the presence of SPaV. A mix of pollen from eight infected plants was used for pollination to examine pollen transmission of SPaV. RT-PCR was utilized for virus detection in these two experiments.

The coat protein gene of SPaV was PCR amplified using oligonucleotide primers CP exp. F / R (Table 1) introducing NdeI and XhoI sites the 5'and 3' terminus of the gene, respectively. The product was gel purified after digestion utilizing the RNAaid kit (Bio 101, Carlsbad CA) and ligated into pET21b® expression vector (Novagen, Madison, WI) that had also been digested with the same enzymes. The recombinant plasmid was transformed into Epicurian Coli BL 21-CodonPlus® cell line (Strategene, La Jolla, CA). After sequencing of the insert to confirm in-frame insertion the protein was expressed as described elsewhere (Tzanetakis et al, 2004) and purified utilizing the Talon CellThru® columns (Clontech, Palo Alto, CA). The purified protein was used for injections in rabbits and mice.

Double and triple antibody sandwich enzyme linked immunosorbent assay (DAS/TAS-ELISA) and tissue blot immunoassay (TBIA) were performed for immunological detection of SPaV as described previously (Converse and Martin 1990;

Tzanetakis 1998).

RESULTS AND DISCUSSION

All but one of the 29 diseased plants from Maryland, the two from California and the seven isolates from the NCGR gave amplicons with primers developed from the heat shock protein 70-homolog (HSP70h) gene. Although several sets of primers from different genes of the virus were utilized, all attempts were unsuccessful to obtain an amplicon from plant M29. The cloned and sequenced dsRNA from M29 revealed the plant was infected with BPYV which caused pallidosis symptoms when grafted onto indicator plants (Fig. 1). About 25% of the field plants were doubly infected with SPaV and BPYV.

The complete sequences of BPYV and SPaV are deposited in the GenBank under accession Nos AY330918-9 and AY488137-8, respectively. BPYV RNA 1 and 2 are 8007 and 7904 nucleotides long respectively, while for SPaV the RNA 1 and 2 are 8067 and 7979 nucleotides, respectively. The 3' UTR of both RNAs of each virus showed significant conservation with the 3' UTRs of BPYV having 86% identity and SPaV 67% identity. This similarity may be involved in polymerase template recognition. RNA 1, ORF 1a, has the characteristic papain-like cysteine protease at the N' terminus that all closteroviruses encode, followed by methyltransferase and helicase motifs. There are two transmembrane domains in the region between the methyltransferase and helicase domain that may be involved in anchoring the replication complex to membranes (Erokhina et al., 2001). The polymerase may be expressed by a +1 ribosomal frame shift as both viruses have the sequence UUUGA at the termination of ORF 1a, a sequence that is found in all criniviruses sequenced to date other than *Cucurbit yellow stunting disorder virus* (CYSDV) that has the sequence UUUAG. Both viruses encode small hydrophobic proteins at the end of RNA 1. SPaV protein has two transmembrane domains and may be cleaved to release a signal peptide while BPYV protein has a single transmembrane domain.

RNA 2 encodes eight ORFs similar to those encoded by other criniviruses with the exception of a small ORF of 6 KDa downstream of the HSP70h gene with marginal homology to the C' terminus of *Little cherry virus* 1 HSP 70h. SPaV encodes the largest structural protein for a filamentous plant virus known to-date, the minor coat protein has an estimated molecular weight of 80 KDa.

Phylogenetic analysis utilizing the polymerase, HSP 70h, and CP genes revealed that BPYV and SPaV are related more closely than any other criniviruses found in the database as of July 2003 (Fig. 3, data not shown for the polymerase and CP).

Results of transmission studies confirmed that SPaV, like BPYV, is transmitted by *T. vaporariorum*. To date transmissions indicate the host range of SPaV includes not only the strawberry and its relatives; *Fragaria ananassa, F. chiloensis, F. vesca, F. virginiana,* but also *Nicotiana benthamiana, N. clevelandii, Physalis wrighii,* and *Urtica californica.* More than 200 seedlings and 70 pollinated plants have been tested for the presence of SPaV and all plants tested negative for SPaV.

Immunological detection of SPaV was unsuccessful utilizing ELISA at all times, while TBIA was sensitive enough for detection during late fall, winter and spring (Fig. 2). We are currently developing monoclonal antibodies that may be useful for detection of SPaV by ELISA or by TBIA throughout the year.

Pallidosis disease has been an underestimated problem in strawberry producing areas of the United States. Preliminary studies have indicated that SPaV is the predominant virus in California and Maryland reaching infection rates that can exceed 70%. The highest infection rate for BPYV was in the Watsonville area of California where more than 20% of plants tested were infected (Tzanetakis et al. 2003). An incidence that was about the same as the aphid-borne viruses *Strawberry mild yellow edge* and *Strawberry crinkle viruses* found in the same fields. Both of the criniviruses were found at low levels in Oregon and Washington, while a limited survey in British Columbia, Canada failed to identify any plants infected with either of the viruses. Future

studies will focus on pollen and seed transmission of SPaV, epidemiology of both viruses in strawberry, determination of acquisition and transmission efficiency of the vector(s), interaction of both viruses in mixed infections with aphid-borne viruses and development of infectious clones of the virus for gene function studies.

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Tables

Table 1. Oligonucleotide primers used for detection of BPYV and SPaV and expression of the coat protein gene (CP). The CP primers underlined sequence are the restriction nuclease recognition sequence while the bold indicates gene sequence.

Primer name	Nucleotide sequence 5'- 3'
Detection primers	
SP 44F	GTGTCCAGTTATGCTAGTC
SP 44R	TAGCTGACTCATCAATAGTG
BP CPm F	TTCATATTAAGGATGCGCAGA
BP CPm R	TGAAAGATGTCCRCTAATGATA
CP modification and sequencing primers	
CP exp. F	ACGCACAGTCAT**ATGGCTGAAACAACCG**
CP exp. R	GAGCTACTCGAG**GTTTCCCGCCAATTGA**

Figures

Fig. 1. Symptomatic leaf of a *Fragaria virginiana* 'UC-10' indicator infected with BPYV (left) and healthy control (right).

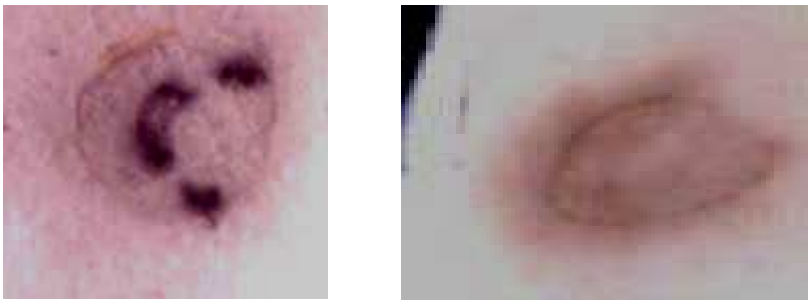

Fig. 2. Tissue blot of a SPaV infected plant (top) and a healthy control (bottom).

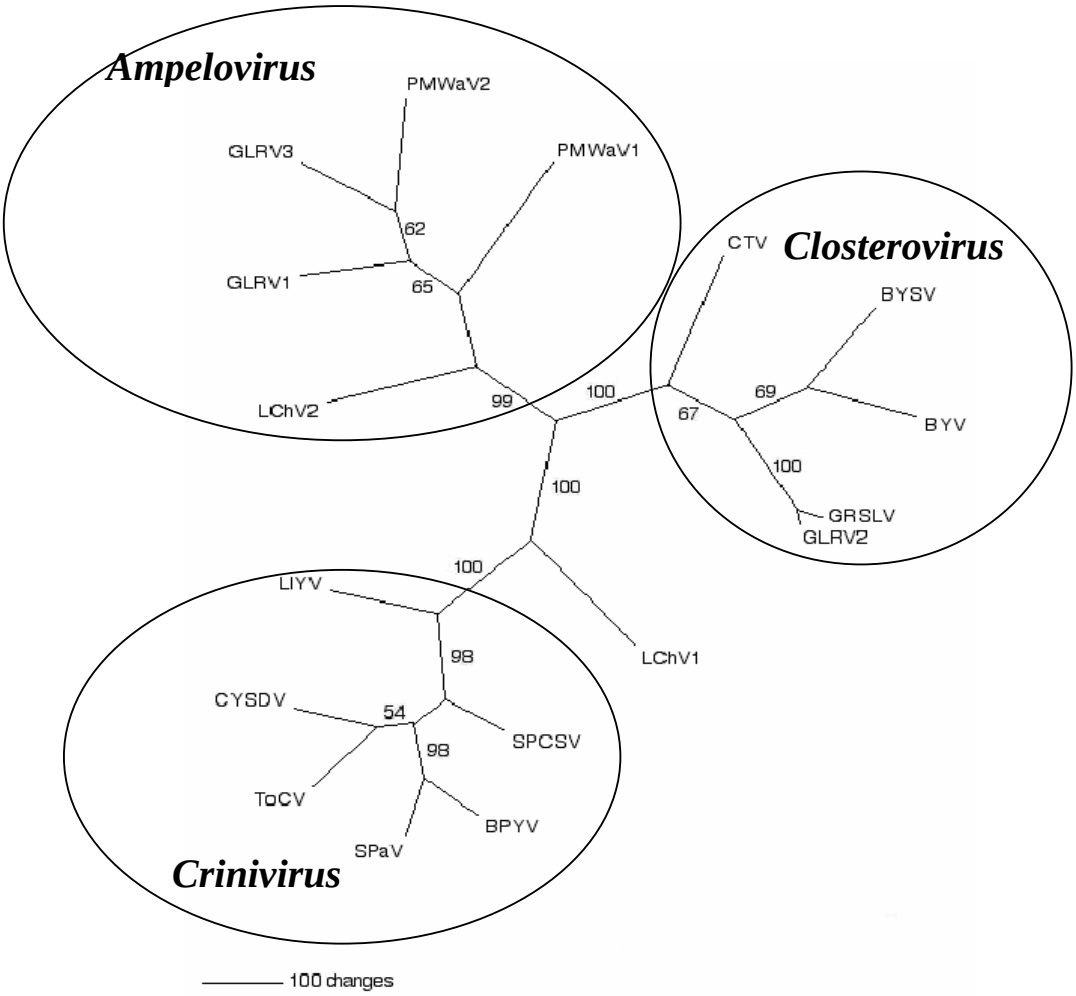

Fig. 3. Phylogram of heat shock protein 70 homolog of SPaV and BPYV and other closteroviruses. Abbreviations and GenBank accession numbers: SPaV, *Strawberry pallidosis associated virus,* AAO92347; BPYV, *Beet pseudo-yellows virus,* AAQ97386; CYSDV, *Cucurbit yellow stunting disorder virus,* NP851572; ToCV, *Tomato chlorosis virus,* AF024630; SPCSV, *Sweet potato chlorotic stunt virus,* NP689401; LIYV, *Lettuce infectious yellows virus,* NP619695; LChV-1, *Little cherry virus-1,* NP045004; CTV, *Citrus tristeza virus,* NP042864; BYSV, *Beet yellow stunt virus,* AAC55662; BYV, *Beet yellows virus,* NP041872; GRSLV, *Grapevine rootstock stem lesion associated virus* NP835247, GLRV2, *Grapevine leafroll associated virus-2,* AAR21242; LChV2, *Little cherry virus-2,* AF531505; PMWaV1, *Pineapple mealybug wilt-associated virus-1***,** AAL66711; GLRV1, *Grapevine leafroll associated virus-1,* AAK38612; GLRV3, *Grapevine leafroll associated virus-3,* NP813799; PMWaV2, *Pineapple mealybug wilt-associated virus-2***,** AAG13941. Bootstrap values are shown as percentage value and only the nodes over 50% are labeled. The bar represents 100 amino acid changes over the length of the proteins.